

Enteric pathogen co-infections in the paediatric population from rural communities in the Vhembe District, South Africa

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Background. Enteric pathogens co-infections are a serious health risk in children under the age of 5 years.

Objective. The study aimed to determine the prevalence of diarrhoea-causing pathogens in children suffering from diarrhoea in rural communities of the Vhembe District.

Methods. A cross-sectional study was conducted from July 2014 to June 2015. Diarrhoeal stool specimens ($N=237$) were collected from children attending primary healthcare facilities in rural communities of the Vhembe District. Stools were screened for enteric viral adenovirus 40/41, rotavirus and norovirus pathogens by means of enzyme immuno-assay (EIA) and enteric bacterial *Escherichia coli* spp. (diarrhoeagenic pathotypes), *Shigella* spp., *Salmonella* spp. and *Vibrio* spp. pathogens by means of multiplex polymerase chain reaction.

Results. A total of 59.1% (140/237) were positive for at least one or more enteric pathogens. Enterotoxigenic *E. coli* (ETEC) (27.9%), enteroaggregative *E. coli* (EAEC) (26.8%) and atypical enteropathogenic *E. coli* (EPEC) (17.9%) were frequently detected in children less than 2 years of age. Bacterial-bacteria co-infections were detected in 24.5% ($n=58$) and bacterial-viral co-infections in 14.3% ($n=34$) of the stool specimens.

Conclusion. The findings indicated that enteric pathogen co-infections are major causes of diarrhoea in children less than 2 years of age in the Vhembe District.

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Gastroenteritis in children is a major global problem.^[1] In sub-Saharan Africa, children are 15 times more likely to die from diarrhoeal diseases before the age of 5 years than children living in developed countries.^[2] The Vhembe District in Limpopo Province, South Africa, consists mostly of rural communities that still lack access to clean drinking water and proper sanitation.^[3] Some people living in these rural communities rely mainly on open water sources such as rivers as their source of drinking water, posing a serious health risk to young children.^[3-5]

Diarrhoeal diseases are caused by various bacterial, parasitic and viral pathogens. Among these, diarrhoeagenic *Escherichia coli* (DEC) are frequently recognised enteric pathogens in children throughout the world.^[6-10] DEC pathotypes co-infections with other enteric pathogens are increasingly detected in children less than 5 years of age.^[11-14] Studies suggest that co-infections might result in increased clinical complications in infected children. In rural communities of the Vhembe District, few studies have been conducted on the prevalence of diarrhoeal pathogens in children.^[4,5,15-18] Consequently, data on the prevalence of co-infections have not been documented in these settings, which could be due to cost and the long waiting period for data analysis before treatment starts. The aim of the present study was to determine the prevalence of co-infections of diarrhoea-causing pathogens in children in rural communities of the Vhembe District, South Africa.

Materials and methods

Setting, collection of stool samples and study design

This prospective cross-sectional study was carried out in rural communities of the Vhembe District in Limpopo Province, in the northern part of South Africa. Ethical clearance was obtained from the University of Venda (Ref: SMNS/12/MBY/0212) and the

Department of Health (Ref: 4/2/2). Informed and signed consent forms were obtained from participating parents and/or primary caretakers before collection of stools.

Between July 2014 and June 2015, 237 diarrhoeal stool samples from children under the age of 5 years were collected from primary healthcare facilities in the Vhembe District. Diarrhoea was defined as the passage of ≥ 3 loose stools within 24 hours.

A standard questionnaire was used to record demographic and clinical data of each study participant. Samples were collected by a qualified nurse using a sterile stool container. The specimens were then kept at -20°C until further analysis.

Bacterial analysis

Genomic DNA was extracted from all stool specimens using the QIAamp Fast DNA stool mini kit according to the manufacturer's instructions (Qiagen, Germany). Three published multiplex-PCR (m-PCR) protocols were used in this study.

A m-PCR protocol was used to indicate the presence of *E. coli*, *Salmonella* spp., *Shigella* spp., and *Vibrio* spp. as previously described.^[19] Briefly: the polymerase chain reaction (PCR) was performed in a total volume of 20 μL containing 1 X QIAGEN multiplex PCR master mix (Qiagen, Germany) and consisted of 100 pmol of each primer, 2 μL of the DNA template, and PCR-grade water.

Samples that tested positive for *E. coli* were further analysed using the 11-gene m-PCR as described previously^[20] to detect diarrhoeagenic *E. coli* (DEC) pathotypes, namely: enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC) and enterohaemorrhagic *E. coli* (EHEC).^[20] Each reaction consisted of 1X Qiagen PCR multiplex mix (containing HotstartTaq DNA polymerase, m-PCR buffer and dNTP mix) (Qiagen, USA); 2 μL of the primer mixture,

2 µL of the DNA sample, 1 µL of gapdh (external control) and 5 µL of PCR-grade water.

Samples that tested positive for *Shigella* spp. and *Salmonella* spp. were further analysed using a m-PCR protocol specifically to detect *S. flexneri*, *Shigella* spp., *S. typhimurium* and *S. enteritidis* as described previously.^[19] Each reaction consisted of 1X Qiagen PCR multiplex mix (containing HotstartTaq DNA polymerase, m-PCR buffer and dNTP mix), 2 µL of the primer, 4 µL of DNA sample and 4 µL of PCR-grade water.

All PCR reactions were conducted using a BIORAD Mxycycler thermal cycler. The following PCR conditions were used: initial activation at 95°C for 15 mins, followed by 35 cycles that consisted of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, extension at 68°C for 2 mins and elongation at 72°C for 5 mins.

All PCR products were analysed using 2.5% (w/v) agarose gel containing ethidium bromide (0.5 µg) and TAE buffer (40 mM tris acetate, 2 mM EDTA, pH 8.3). The DNA was electrophoresed for 1 - 2 hrs in an electric field strength of 80 - 100 V, and visualised using UV light (Gene Genius Bio imaging system, Vacutec, Costa Mesa, CA, USA). The relative sizes of the DNA fragments were estimated by comparing their electrophoretic mobility with the standards on each gel using 100 bp markers (Fermentas).

Viral analysis

Viral pathogens were determined using enzyme immunoassays (EIAs). Enteric adenovirus type 40/41 was detected using Premier Adenoclone 40/41 kit (Meridian Bioscience, Europe), rotavirus using Rotavirus EIA kit (R-Biopharm, Germany) and norovirus using RIDASCREEN Norovirus 3rd Generation EIA kit (R-Biopharm, Germany) according to manufacturers' instructions.

Data analysis

Data from the EIA and PCR samples were entered into Microsoft Excel (2010) spreadsheets. Data from questionnaires were entered into separate Excel spreadsheets. The average and median of the samples were calculated using Microsoft Excel (2010) when needed.

Results

Demographic data of study population

A total of 237 diarrhoeal stool specimens were collected from children younger than 5 years in primary healthcare facilities. The median age of the study participants was 10 months (range 0 - 60 months). Most of the study participants were age 0 - 12 months (58.2%, *n*=138) and 12 - 24 months (21.9%, *n*=52). Gender distribution was 122 (51.5%) male and 105 (44.3%) female.

Prevalence of enteric pathogens

At least 1 enteric pathogen was detected in 140 (59.1%) of the diarrhoeal specimens (Table 1). Single diarrhoeagenic *E. coli* (DEC) pathotypes, ETEC 7 (3.0%), EAEC 13 (5.5%) and atypical EPEC 2 (0.8%) were detected. Single viral pathogens detected were rotavirus 9 (3.8%), norovirus 12 (5.1%) and adenovirus 40/41 6 (2.5%) (Table 1).

Mixed infections of 2 or more bacterial pathogens were detected in 58 (24.5%); t-EPEC co-infected with ETEC (5.5%, *n*=13) and ETEC co-infected with EAEC (4.2%, *n*=10) were the most frequent co-infecting pathogens detected (Table 2). Bacterial and viral co-infections were detected in 34 (14.3%); EAEC and rotavirus were detected in 10 (2.5%) of the diarrhoeal specimens (Table 2). Fig. 1 indicates single and mixed pathogens on electrophoresis gel using *E. coli* as positive control.

Children under 12 and 24 months were mostly infected with ETEC (34.8% and 28.8%, respectively) and EAEC (26.0% and 28.8%, respectively) (Table 3). Overall, 190 of the children under the age of 2 years (0 - 24 months) were infected with EAEC (26.8%, *n*=51), ETEC (27.9%, *n*=53) and atypical EPEC (17.9%, *n*=34).

Table 1. Prevalence of enteric pathogens in children less than 5 years of age (N=237)

| Diarrhoeal pathogen | <i>n</i> (%) |
|---|--------------|
| Samples positive | 140 (59.1%) |
| Positive for only one diarrhoeal pathogen | 64 (27.0%) |
| ETEC | 7 (3.0%) |
| EAEC | 13 (5.5%) |
| Atypical EPEC | 2 (0.8%) |
| Typical EPEC | 2 (0.8%) |
| EIEC | 9 (3.8%) |
| <i>Shigella</i> spp. | 2 (0.8%) |
| <i>S. enteritidis</i> | 1 (0.4%) |
| <i>Vibrio</i> spp. | 1 (0.4%) |
| Adenovirus 40/41 | 6 (2.5%) |
| Norovirus | 12 (5.1%) |
| Rotavirus | 9 (3.8%) |
| Bacterial and bacteria co-infections | 58 (24.5%) |
| Bacterial and virus co-infections | 34 (14.3%) |
| Virus and virus co-infections | 2 (0.8%) |
| No pathogen detected | 97 (40.1%) |

ETEC = enterotoxigenic *E. coli*; EAEC = enteroaggregative *E. coli*;
EPEC = enteropathogenic *E. coli*; EIEC = enteroinvasive *E. coli*.

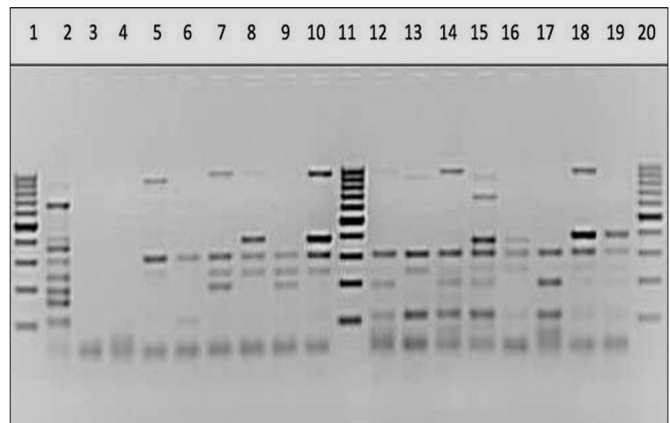


Fig. 1. Agarose gel electrophoresis results for multiplex PCR amplification of diarrhoeagenic *E. coli*. Lane 1, 11 and 20 molecular weight marker; Lane 2 positive control *eae* (917 bp), *stx2* (779 bp), *ial* (630 bp), *bfp* (410 bp), *LT* (330 bp), *Gapdh* (238 bp), *Eagg* (194 bp), *ST* (160 bp), *Asta* (106 bp)); Lane 3 negative control; Lane 4 extraction negative; Lane 5 - 10, 12 - 19 samples that tested positive for single and multiple pathogens.

Other bacterial pathogens such as *S. flexneri* (4.2%, *n*=10), *S. enteritidis* (1.3%, *n*=3) and *S. typhimurium* (0.4%, *n*=1) were detected across the age groups. Adenovirus 40/41 was detected in 10 (7.2%) of the children aged 12 months, 4 (7.7%) of the children aged 24 months and 4 (11.1%) of the children aged 60 months (Table 3).

Of the 48 children infected with t-EPEC, 13 (27.1%) had diarrhoea only, while 36 (75%) had diarrhoea accompanied by other symptoms such as fever (25.0%, *n*=12), vomiting (25.0%, *n*=12) and dehydration (6.3%, *n*=3) (Table 4). Children infected with ETEC and a-EPEC (41.0% and 48.6%, respectively) had watery stools while those infected with EAEC had formed stools (53.2%, *n*=33) (Table 4).

Discussion

In this study, the prevalence of enteric pathogens was investigated in children suffering from diarrhoea in rural communities of the Vhembe District, South Africa. The prevalence rate of enteric

Table 2. Frequency of co-infecting pathogens in children less than 5 years of age (N=237)

| Combination of pathogens | n (%) | Combination of pathogens | n (%) |
|--|------------|---|------------|
| Bacterial and bacteria co-infections | 58 (24.5%) | Bacterial and virus co-infections | 34 (14.3%) |
| ETEC + typical EPEC | 13 (5.5%) | EAEC + rotavirus | 6 (2.5%) |
| EAEC + ETEC | 10 (4.2%) | EAEC + adenovirus 40/41 | 1 (0.4%) |
| EAEC + typical EPEC | 5 (2.1%) | EAEC + norovirus | 1 (0.4%) |
| ETEC + EHEC | 1 (0.4%) | ETEC + adenovirus 40/41 | 1 (0.4%) |
| ETEC + atypical EPEC | 4 (1.7%) | <i>Shigella</i> spp. + rotavirus | 1 (0.4%) |
| EAEC + atypical EPEC | 1 (0.4%) | atypical EPEC + EIEC + rotavirus | 1 (0.4%) |
| EIEC + typical EPEC | 1 (0.4%) | ETEC + typical EPEC + norovirus | 4 (1.7%) |
| ETEC + <i>S. flexneri</i> | 2 (0.8%) | ETEC + atypical EPEC + rotavirus | 1 (0.4%) |
| EAEC + <i>S. flexneri</i> | 1 (0.4%) | EIEC + rotavirus | 1 (0.4%) |
| Atypical EPEC + <i>S. flexneri</i> | 1 (0.4%) | Typical EPEC + norovirus | 1 (0.4%) |
| <i>S. flexneri</i> + <i>S. enteritidis</i> | 1 (0.4%) | EAEC + atypical EPEC + norovirus | 1 (0.4%) |
| EAEC + ETEC + typical EPEC | 7 (3.0%) | EAEC + atypical EPEC + adenovirus 40/41 | 1 (0.4%) |
| ETEC + t-EPEC + EIEC | 1 (0.4%) | Atypical EPEC + rotavirus + norovirus | 1 (0.4%) |
| EAEC + ETEC + EIEC | 1 (0.4%) | EIEC + rotavirus + adenovirus 40/41 | 1 (0.4%) |
| EAEC + ETEC + atypical EPEC | 4 (1.7%) | EAEC + ETEC + atypical EPEC + norovirus | 2 (0.8%) |
| EAEC+ atypical EPEC + EIEC | 1 (0.4%) | EAEC + ETEC + typical EPEC + adenovirus 40/41 | 3 (1.3%) |
| EAEC+ ETEC + atypical EPEC | 2 (0.8%) | ETEC + atypical EPEC + <i>S. flexneri</i> + rotavirus | 1 (0.4%) |
| EAEC + typical EPEC + <i>S. flexneri</i> | 1 (0.4%) | EAEC + ETEC + EIEC + adenovirus 40/41 | 1 (0.4%) |
| ETEC + typical EPEC + <i>S. flexneri</i> | 1 (0.4%) | EAEC + ETEC + EHEC + norovirus | 1 (0.4%) |
| Virus and virus co-infections | 2 (0.8%) | ETEC + atypical EPEC + adenovirus 40/41 | 1 (0.4%) |
| Rotavirus + norovirus | 1 (0.4%) | EIEC + atypical EPEC + EIEC + norovirus | 1 (0.4%) |
| Rotavirus + adenovirus 40/41 | 1 (0.4%) | EAEC + <i>S. flexneri</i> + adenovirus 40/41 | 1 (0.4%) |
| | | Typical EPEC + <i>S. enteritidis</i> + <i>S. typhimurium</i> + adenovirus 40/41 | 1 (0.4%) |

EAEC = enteroaggregative *E. coli*; ETEC = enterotoxigenic *E. coli*; EHEC = enterohaemorrhagic *E. coli*; EPEC = enteropathogenic *E. coli*; EIEC = enteroinvasive *E. coli*.

Table 3. Distribution of diarrhoea-causing pathogens based on age of population

| Pathogen | All age groups (N=237) | Age group (months) | | | |
|-----------------------|---------------------------|------------------------|------------------------|------------------------|-----------------------|
| | | 0 - 12 (n=138, 58%) | 12 - 24 (n=52, 22%) | 24 - 60 (n=36, 15%) | No data (n=11, 5%) |
| Bacterial pathogen | | | | | |
| EAEC | 62 (26.1%) | 36 (26.0%) | 15 (28.8%) | 9 (25.0%) | 2 (18.1%) |
| Typical EPEC | 48 (20.3%) | 31 (22.5%) | 9 (17.3%) | 8 (22.2%) | 0 (0%) |
| Atypical EPEC | 37 (15.6%) | 25 (18.1%) | 9 (17.3%) | 3 (8.3%) | 0 (0%) |
| ETEC | 73 (30.8%) | 48 (34.8%) | 15 (28.8%) | 9 (25.0%) | 1 (9.1%) |
| EIEC | 18 (7.6%) | 7 (5.1%) | 4 (7.7%) | 7 (19.4%) | 0 (0%) |
| EHEC | 2 (0.8%) | 0 (0%) | 1 (1.9%) | 0 (0%) | 1 (9.1%) |
| <i>S. flexneri</i> | 10 (4.2%) | 4 (2.9%) | 3 (5.8%) | 3 (8.3%) | 0 (0%) |
| <i>Shigella</i> spp. | 3 (1.3%) | 1 (0.7%) | 0 (0%) | 2 (5.6%) | 0 (0%) |
| <i>S. typhimurium</i> | 1 (0.4%) | 0 (0%) | 1 (1.9%) | 0 (0%) | 0 (0%) |
| <i>S. enteritidis</i> | 3 (1.3%) | 2 (1.4%) | 1 (1.9%) | 0 (0%) | 0 (0%) |
| <i>Vibrio</i> spp. | 1 (0.4%) | 1 (0.7%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Viral pathogen | | | | | |
| Adenovirus 40/41 | 18 (7.6%) | 10 (7.2%) | 4 (7.7%) | 4 (11.1%) | 0 (0%) |
| Norovirus | 21 (8.9%) | 11 (8.0%) | 5 (9.6%) | 1 (2.7%) | 4 (36.4%) |
| Rotavirus | 24 (10.1%) | 7 (5.1%) | 9 (17.3%) | 5 (13.8%) | 3 (27.2%) |

EAEC = enteroaggregative *E. coli*; EPEC = enteropathogenic *E. coli*; ETEC = enterotoxigenic *E. coli*; EIEC = enteroinvasive *E. coli*; EHEC = enterohaemorrhagic *E. coli*.

pathogens in this study was 59.1%. Of all the diarrhoeal pathogens detected, diarrhoeagenic *E. coli* (DEC) pathotypes were more prevalent, with ETEC (30.8%) and EAEC (26.1%) being detected

at high rates. Other studies have also reported on the prevalence of EAEC in countries such as Mozambique,^[8] Kenya,^[10] India,^[21] Sudan^[9] and Vietnam^[7] as a major cause of moderate to severe diarrhoea in

Table 4. Clinical symptoms of children infected with diarrhoeagenic *E. coli* pathotypes (N=237)

| Properties | EAEC (n=62) | Atypical EPEC (n=37) | Typical EPEC (n=48) | EPEC (n=73) | EIEC (n=18) | EHEC (n=2) |
|---------------------------------|----------------|-------------------------|------------------------|----------------|----------------|---------------|
| Clinical symptoms | | | | | | |
| Diarrhoea only | 10 (16.1%) | 18 (48.6%) | 13 (27.1%) | 27 (37.0%) | 5 (27.8%) | 0 (0%) |
| Diarrhoea/other symptoms | 52 (83.9%) | 19 (51.4%) | 36 (75.0%) | 46 (63.0%) | 13 (72.2%) | 2 (100%) |
| Abdominal cramps | 8 (12.9%) | 4 (10.8%) | 9 (18.8%) | 11 (15.1%) | 4 (22.2%) | 1 (50%) |
| Fever $\geq 38^{\circ}\text{C}$ | 16 (25.8%) | 6 (16.2%) | 12 (25.0%) | 17 (23.3%) | 3 (16.7%) | 1 (50%) |
| Vomiting | 19 (30.6%) | 8 (21.6%) | 12 (25.0%) | 13 (17.8%) | 4 (22.2%) | 0 (0%) |
| Dehydration | 9 (14.5%) | 1 (2.7%) | 3 (6.3%) | 5 (6.8%) | 2 (11.1%) | 0 (0%) |
| Stool type | | | | | | |
| Watery | 19 (30.6%) | 18 (48.6%) | 20 (41.7%) | 30 (41.0%) | 9 (50.0%) | 1 (50%) |
| Loose/mushy | 10 (16.1%) | 5 (13.5%) | 10 (20.8%) | 13 (17.8%) | 6 (33.3%) | 0 (0%) |
| Formed | 33 (53.2%) | 14 (37.8%) | 19 (39.6%) | 30 (41.0%) | 3 (16.7%) | 1 (50%) |

EAEC = enteroaggregative *E. coli*; EPEC = enteropathogenic *E. coli*; ETEC = enterotoxigenic *E. coli*; EIEC = enteroinvasive *E. coli*; EHEC = enterohaemorrhagic *E. coli*.

children under 2 years. ETEC has been reported to cause diarrhoea in children under 5 years.^[8,13,22] EPEC has been recognised as a diarrhoea-causing pathogen in children less than 2 years,^[6,14,23] and is reported to cause deaths in infants.^[2,24] EPEC is characterised mainly by the presence or absence of the bundle-forming pili (*bfp*). In the present study, typical EPEC of 20.3% (with *bfp* and *eae*) was higher when compared with atypical EPEC of 15.6% (no *bfp*, only *eae* gene) across all age groups. The Global Multicenter Study (GEMS) which focused on major countries located in Asia and sub-Saharan Africa also reported ETEC, typical EPEC, rotavirus and *Shigella* as some of the major causes of moderate to severe diarrhoea in children under the age of 2 years.^[2]

Enteric viruses such as rotavirus were detected in 10.1% of samples as compared with other studies,^[25-28] which could mainly be due to the introduction of the rotavirus vaccine in South Africa.^[29] Adenovirus 40/41 and norovirus were detected in low frequencies across all age groups. Other studies have reported these viruses cause diarrhoea in children, especially under the age of 5 years.^[23,26,28,30] The low detection rate of viruses has led to children, especially those under 2 years of age, to be susceptible to other enteric pathogens as seen in this study. One of the limitations in this study due to financial constraints, was the use of EIAs for detection of enteric viruses instead of PCR. As PCR is more sensitive than EIA, commercially available EIA cannot detect certain types of viral recombinant genes^[31] and, as a result, enteric viruses were detected in a limited number of samples. Another limitation was that demographic and clinical data of study participants were not consistently collected by some of the professional nurses as some of the data were missing.

Conclusion

Children living in rural settings of the Vhembe District have been previously reported to be infected with diarrhoeal pathogens such as norovirus,^[18] rotavirus,^[5,17] EAEC,^[4,15] ETEC, EPEC,^[4] *Shigella* spp. and *Salmonella* spp.^[5] In the present study, children under the age of 24 months were found to be more susceptible to enteric infections, with an occurrence of 2 or more pathogens detected in most of the

study participants. DEC pathotypes occurred frequently in mixed infections with other bacterial and viral pathogens. There are several studies that have reported on the occurrence of co-infecting pathogens in children.^[12,16,30,32] The most frequent DEC co-infection was ETEC and typical EPEC in 5.5% of the episodes. Crane *et al.*^[33] studied the interaction of EPEC and ETEC *in vitro* and found that ETEC virulence genes were significantly increased when co-infected with EPEC.

The high detection rate of DEC pathotypes, especially in co-infections, raises serious health concerns for the well-being of young children. Further studies are therefore needed to determine the interaction of DEC pathotypes and the role that these pathogens play in severity of disease during co-infections, as this has not been well defined. Clinical symptoms of all the DEC infections were almost similar, and most cases of diarrhoea were accompanied by other symptoms such as abdominal pain, vomiting, fever and dehydration. In conclusion, enteric pathogens are important aetiological agents of diarrhoea in children. DEC infections were more prevalent, posing a health risk to children less than 2 years of age in rural communities of the Vhembe District of Limpopo Province, South Africa.

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Author contributions. NP was the project leader and supervisor of SEL. ANT was co-supervisor and mentor. JPK and SEL collected samples. SEL and TGB did the molecular analyses. SEL did the ELISA analysis and concept manuscript. All authors contributed to the writing and approval of the manuscript.

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